Immunology
The Journal of cells, molecules, systems and technologies

British Society for immunology

IMMUNOLOGY ORIGINAL ARTICLE

Negative regulation of Nod-like receptor protein 3 inflammasome activation by T cell Ig mucin-3 protects against peritonitis

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doi:10.1111/imm.12812
Received 8 March 2017; revised 13 July 2017; accepted 31 July 2017.
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Summary

The Nod-like receptor protein 3 (NLRP3) inflammasome plays roles in host defence against invading pathogens and in the development of autoimmune damage. Strict regulation of these responses is important to avoid detrimental effects. Here, we demonstrate that T cell Ig mucin-3 (Tim-3), an immune checkpoint inhibitor, inhibits NLRP3 inflammasome activation by damping basal and lipopolysaccharide-induced nuclear factor-κB-mediated up-regulation of NLRP3 and interleukin-1β during the priming step and basal and ATP/lipopolysaccharide-induced ATP production, K^+ efflux, and reactive oxygen species production during the activation step. Residues Y256/Y263 in the C-terminal region of Tim-3 are required for these inhibitory effects on the NLRP3 inflammasome. In mice with alum-induced peritonitis, blockade of Tim-3 exacerbates peritonitis by overcoming the inhibitory effect of Tim-3 on NLRP3 inflammasome activation, while transgenic expression of Tim-3 attenuates inflammation by inhibiting NLRP3 inflammasome activation. Our results show that Tim-3 is a critical negative regulator of NLRP3 inflammasome and provides a potential target for intervention of diseases with uncontrolled inflammasome activation.

Keywords: macrophage; NLRP3 inflammasome; Tim-3.

Introduction

The Nod-like receptor protein 3 (NLRP3) inflammasome, a cytosolic protein complex composed of NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and caspase-1, controls caspase-1 activation in, and the release of the pro-inflammatory cytokine interleukin-1β (IL-1β) by, macrophages.[1–3]. It is an important innate immune sensor that can be activated in response to structurally diverse pathogen-associated molecular patterns, such as lipopolysaccharide (LPS), and damage-associated molecular patterns, such as toxins, ATP and cholesterol crystals.[4,5] Activation of the NLRP3 inflammasome contributes to the development of immune responses and host defence. However, excessive NLRP3 inflammasome activation is involved in many

Abbreviations: FBS, fetal bovine serum; IL-1β, interleukin-1β; LPS, lipopolysaccharide; mAb, monoclonal antibody; NF-κB, nuclear factor-κB; NLRP3, Nod-like receptor protein 3; PEC, peritoneal exudate cell; PLF, peritoneal lavage fluid; ROS, reactive oxygen species; STAT-1, signal transducer and activator of transcription 1; Tim-3, T cell Ig mucin-3; WT, wild-type

inflammatory diseases, including arthritis, atherosclerosis and peritonitis.[6–8] To avoid the deleterious consequences of its inappropriate activation, negative regulator(s) of the NLRP3 inflammasome with therapeutic potential are now under intensive investigation.[9–11]

NLRP3 inflammasome activation is tightly regulated at different levels.[12] A priming signal (referred to as step 1) up-regulates expression of NLRP3 and the inflammasome substrate pro-IL-1β by activating the pro-inflammatory transcription factor nuclear factor-κB (NF-κB).[13,14] The activation signal (step 2) involves a mechanism that has not yet been defined, but has been suggested to include a decrease in the intracellular concentration of K^+ and the production of reactive oxygen species (ROS).[15,16] Multiple regulatory mechanisms, such as type I interferon,[9] nitric oxide,[11] TRIM30[15] and the aryl hydrocarbon receptor,[17] can attenuate NLRP3 inflammasome activation. All of these identified regulators operate within the cell and at one of the two steps of NLRP3 inflammasome activation (step 1 or 2), so new regulators with more widespread activities are of great interest.

T cell Ig mucin-3 (Tim-3) is an immune checkpoint inhibitor that was first identified on T effector cells, including T helper type 1, T helper type 17 and Tc1 cells, and induces tolerance of these T effector cells.[18] Recent studies, including our own, have shown that Tim-3 also functions as a checkpoint inhibitor for innate immune cells and plays critical roles in maintaining the homeostasis of innate immunity.[19,20] For example, dysregulated Tim-3 expression on macrophages and dendritic cells is associated with immune disorders, such as ulcerative colitis, sepsis and tumours.[21–24] Although it is known that Tim-3 is involved in the physiopathology of innate immunity-mediated disorders and shows therapeutic potential, its roles in the regulation of inflammasomes remain largely unclear. In addition, how Tim-3 signals in innate immune cells remains to be determined.

In this study, we demonstrated that, in contrast to the regulators described above that function at only one step of NLRP3 inflammasome activation, Tim-3 signalling acts at both steps, acting at the priming step by inhibiting NF-κB and at the activation step by inhibiting ATP release, K^+ efflux and ROS production. *In vivo*, Tim-3 signalling inhibits NLRP3 inflammasome activation and protects mice from peritonitis. We have therefore identified a new negative regulator of NLRP3 inflammasome-mediated inflammatory reactions with therapeutic potential.

Materials and methods

Study population

The study was approved by the Ethics Committee of the General Hospital of the PLA, Beijing, China. All protocols were performed in accordance with relevant guidelines and regulations of the Declaration of Helsinki and all patients gave their written informed consent for the study. The study population comprised two groups of subjects, healthy volunteers (n = 8, aged from 20 to 50 years) and peritonitis patients (n = 8, aged from 20 to 50 years), from whom sera were obtained and used for ELISA examination.

Mice

Male C57BL/6 mice (6 to 8 weeks old) were obtained from Jackson Laboratory (Bar Harbor, ME). Tim-3 transgenic mice were generated in the Transgenic Core Facility of Cyagen Biosciences Inc., Guangzhou, China by overexpressing Tim-3 under the control of the cytomegalovirus promoter; incorporation was confirmed by PCR and Tim-3 expression on macrophages and other cells was confirmed using flow cytometry.[25] All mice were bred and maintained in our facilities under specific pathogen-free conditions. All treatment of mice in this study was in strict compliance with the guidelines for the care and use of laboratory animals set out by the Beijing Institute of Basic Medical Sciences, and the protocol was approved by the Committee on the Ethics of Animal Experiments of the Beijing Institute of Basic Medical Sciences.

Reagents

The recombinant fusion protein sTim-3-Ig was prepared by fusing cDNA coding for the soluble extracellular domain of mouse Tim-3 to that coding for the single-chain Fc fragment of human IgG1 in the pet28a+ vector and expression in *Escherichia coli* BL21 as described previously.[20] The presence and purity of sTim-3-Ig were confirmed by SDS–PAGE and Western blot analysis using rabbit anti-mouse Tim-3 antibodies (Abcam, Cambridge, UK). The Fc fragment of human IgG1 (Ig) was prepared and purified from *E. coli* BL-21 in an identical manner and used as the negative control. The endotoxin concentration in both sTim-3-Ig and Ig was less than 1·0 EU/mg. LPS (*E. coli* 055:B5), ATP (A6419) and the ROS-specific inhibitor *N*-acetylcysteine (NAC; A9165), NLRP3 inhibitor MCC950, P2X7 ATP receptor inhibitor and signal transducer and activator of transcription 1 (STAT1) inhibitor Fludarabine were from Sigma-Aldrich (St Louis, MO). Imject alum (77161) was purchased from Thermo Scientific (Waltham, MA). The NF-κB inhibitor Bay11-7082 was from Santa Cruz Biotechnology (Dallas, TX). The IL-1β and IL-6 ELISA kits were from eBioscience (San Diego, CA) and the sTim-3 ELISA kit was from Sino Biologicals Inc. (Beijing, China).

Antibodies

The rabbit anti-mouse antibodies used were anti-caspase-1(p20) (Adipogen; AG-20B-0042), anti-NLRP3 (D4D8T),

anti-NF-κB p65 (C22B4), anti-phospho-κB p65 (S536) and anti-tubulin (Cell Signaling, San Diego, CA), and anti-pro-IL-1β (Abclonal; A1112). For flow cytometry, allophycocyanin-conjugated rat anti-mouse CD11b(M1/70), FITC-conjugated rat anti-mouse Ly-6G monoclonal antibody (mAb) (1A8), phycoerythrin-conjugated rat anti-mouseLy-6C mAb (HK1.4),anti-mouse F4/80 mAb (BM8) and phycoerythrin-conjugated rat anti-mouse Tim-3 mAb(GL3) were all from eBioscience.

Cell culture and transfection

The mouse macrophage cell lines RAW264.7and J774 were obtained, respectively, from the American Type Culture Collection (Manassas, VA) and China Infrastructure of Cell Line Resources. Mouse peritoneal macrophages were prepared as described previously.[20] All cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml of penicillin and 100 U/ml of streptomycin (all from Sigma Aldrich) in a humidified 5% CO_2 atmosphere at 37°C.

For cell transfection, Tim-3 cDNA was cloned into pcDNA3.1 to generate Tim-3-wt, and the empty vector pcDNA3.1 was used as the control. Overlap PCR was used to generate the point mutation construct Y256A/Y263A-Tim-3, in which the indicated tyrosine residues in the tail region of Tim-3 were replaced by alanine. Lipofectamine 2000 was used for transient transfection. J774 cells were transiently transfected in six-well plates with 4 ng of plasmid, then, 42 hr later, were used in studies.

ELISA

Interleukin-1β, IL-6, and sTim-3 levels in cell-free supernatants were measured using a sandwich ELISA according to the manufacturer's protocol.

Quantitative real-time RT-PCR

Gene expression was analysed by two-step quantitative RT-PCR. Total RNA was extracted from RAW264.7 cells, J774 cells or mouse peritoneal lavage fluid (PLF) -derived cells using TRI Reagent (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. RNA (0·2–1 μg) was reverse transcribed in a 20-μl reaction volume (42°C, 30 min; 95°C, 5 min), using a QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany); then cDNA (2 μl) was amplified using SYBR Green I Master Mix (Roche, Basel, Switzerland) and a LightCycler 480 PCR System (Roche). All tests were carried out on duplicate 20-μl reaction mixtures in 96-well plates, and a negative control with no cDNA template was included in each run. The specificity of the products was confirmed by visual inspection of the melting curves, and the products were run on a 1·2% agarose gel. The relative expression of a gene was determined using the 2^{-DDCt} method, with GAPDH as the internal control. The mRNA levels in each sample were determined after correction for GAPDH expression. The gene-specific threshold cycle (Ct) for each sample (△Ct) was corrected by subtracting the Ct for the GAPDH housekeeping gene. The primer sequences for the targeted cDNAs are listed in Western blots (Table 1).

Western blotting was performed to measure levels of NLRP3, Pro-caspase-1, caspase1-p20 (casp1-p20), NF-κB p65, phospho-NF-κB p65 and tubulin. Briefly, 50 μg of protein was electrophoretically separated on a 12% SDS–polyacrylamide gel and transferred to a PVDF membrane, which was then blocked by incubation for 1 hr at room temperature in 5% fat-free dry milk in Tris-buffered saline containing 0·05% Tween 20 (TBST). The blots were then incubated overnight at 4°C with a 1 : 1000 dilution of rabbit antibodies against casp1-p20, NF-κB p65, phospho-NF-κB p65 or tubulin diluted in TBST containing 5% bovine serum albumin, washed for 25 min with TBST, and incubated for 1 hr at room temperature with alkaline phosphatase-conjugated anti-rabbit IgG antibodies (KPL, Gaithersburg, MD) (1:20 000 in TBST containing 5% bovine serum albumin); then bound antibodies were visualized using ECL kits (Amersham Biosciences, Chalfont St Giles, UK).

FACS analysis and macrophage sorting

Cells harvested from the PLF were collected and stained with CD11b mAb,Ly-6G mAb and Ly-6C mAb diluted in

Table 1. Sequences of the primers used for real-time PCR

	Sense primer	Antisense primer
mNLRP3	5′-TGGGTTCTGGTCAGACACGAG-3′	5′-GTCATTCCACTCTGGCTGGT-3′
mIL-1β	5′-GCCCATCCTCTGTGACTCA-3′	5′-AGGCCACAGGTATTTTGTC-3′
mTim-3	5′-GACCCTCCATAATAACAA-3′	5′-TAATAAGGCTCAAACTCG-3′
hNLRP3	5′-AGAAGCTCTGGTTGGTCAGC-3′	5′-CAAGGCATTCTCCCCCACAT-3′
hIL-1β	5′-AACCTCTTCGAGGCACAAGG-3′	5′-GGCGAGCTCAGGTACTTCTG
mGAPDH	5′-TCTTGGGCTACACTGAGGAC-3′	5′-CATACCAGGAAATGAGCTTGA-3′
hGAPDH	5′-AATGGAAATCCCATCACCATCT-3′	5′-CGCCCCACTTGATTTTGG-3′

2% FBS in PBS; Isotype control antibodies (eBioscience) were used as controls. After two washes with PBS/2% FBS. Ly-6C monocytes($CD11b^+$ $Ly6C^+$) and neutrophils ($CD11b^+$ $Ly6G^+$) were analysed by flow cytometry in a FACSCalibur (BD Biosciences). For macrophage collection, antibodies against mouse F4/80 and CD11b were used to isolate macrophages from PLF using FACS cell-sorting methods.

ROS measurement

Intracellular ROS was measured using the the ROS-specific fluorescent probe DCFH-DA (Beyotime Biotechnology, Shanghai, China; S0033) according to the manufacturer's protocol.

ATP measurement

Extracellular ATP was measured using an ATP Assay kit (Beyotime Biotechnology, S0026) according to the manufacturer's instructions. The luminescence produced was measured with a luminometer counter (Perkin Elmer Wallac Victor 3 1420; Perkin Elmer, Waltham, MA), and the concentration of ATP was calculated using an ATP standard curve.

Measurement of caspase-1 activity

Caspase-1 activity was measured using a colorimetric assay (Applygen Technologies Inc., Beijing, China) and a substrate (YVAD-*p*NA) specific for this enzyme. The results are expressed as a fold value for treated cells compared with untreated control cells.

In vivo peritonitis model

Male C57BL/6J or Tim-3-TG mice (6 to 8 weeks old) were injected intraperitoneally with 700 μg of alum (Thermo) as described before,[8,9,17] and, with 200 μg of sTim-3-Ig or Ig control, then, after 12 hr, the peritoneal cavity was washed with 6 ml of cold PBS and the PLF was collected and peritoneal exudate cells (PECs), neutrophils and monocytes were counted by fluorescence-activated cell sorting, while the supernatants were concentrated using an Amicon Ultra 10K (Millipore, Billerica, MA) for ELISA analysis.

Statistical analysis

Data are expressed as the mean ± SD. Differences between groups were analysed using the Kruskal–Wallis test and analysis of variance. A P-value $< 0{\cdot}05$ was considered significant and SPSS software (version 20·0) was used for all statistical procedures.

Results

Tim-3 inhibits NLRP3 inflammasome activation

To test whether Tim-3, a checkpoint inhibitor for innate immune cells, is involved in inflammasome regulation, we examined the effects of Tim-3 over-expression or Tim-3 blockade on NLRP3 inflammasome activation in macrophages. Caspase-1 status was monitored by the appearance of the p20 cleavage product and by directly measuring caspase-1 activity. As LPS or ATP alone cannot activate NLRP3 inflammasome in and induce IL-1β secretion by macrophages [8], here we use LPS plus ATP to activate NLRP3 inflammasome. When peritoneal macrophages isolated from wild-type (WT) or Tim-3 transgenic (Tim-3-TG) mice were stimulated sequentially with LPS and ATP, the active caspase-1 subunit p20 (casp1-p20) increased but Tim-3 over-expression was shown to result in decreased levels of casp1-p20 (Fig. 1a) and significantly decreased caspase-1 activity (Fig. 1b). As IL-1β is a pro-inflammatory cytokine cleaved by the NLRP3 inflammasome, we also examined the effects of Tim-3 over-expression on IL-1β secretion by macrophages. As shown in Fig. 1(c), Tim-3 over-expression significantly inhibited LPS/ATP-induced IL-1β secretion by macrophages. These data show that Tim-3 signalling can inhibit NLRP3 inflammasome activation. Soluble Tim-3 is used to competitively inhibit the interaction between membrane Tim-3 and its ligands.[20] To test this in another way, soluble Tim-3 protein was used to block Tim-3 signalling in J774, a mouse macrophage cell line, and resulted in increased levels of casp1-p20 (Fig. 1d), increased caspase-1 activity (Fig. 1e) and increased IL-1β secretion (Fig. 1f) compared with addition of Ig control. The Western blot results in (Fig.1a, d) were quantified by densitometry and are shown in the Supplementary material (Fig. S1a–d).As we reported before, Tim-3 also regulates the expression of tumour necrosis factor-α and IL-6.[25] To test whether Tim-3 signalling inhibits IL-1β especially through NLRP3, here NLRP3 inhibitor MCC-950 was used and we found that NLRP3 inhibition reversed soluble Tim-3-increased production of IL-1β (Fig.1g) but not that of tumour necrosis factor-α (see Supplementary material, Fig. S1e). These data demonstrate that Tim-3 functions as a negative regulator of NLRP3 inflammasome activation.

Tim-3 inhibits NLRP3 and IL-1β expression in macrophages by inhibiting NF-κB activity

Based on the 'two-step' model of NLRP3 inflammasome activation, we first examined the effects of Tim-3 on the priming signal (signal 1) for the NLRP3 inflammasome. The results in Fig. 2 show that transgenic over-expression of Tim-3 in peritoneal macrophages from Tim-3-TG mice

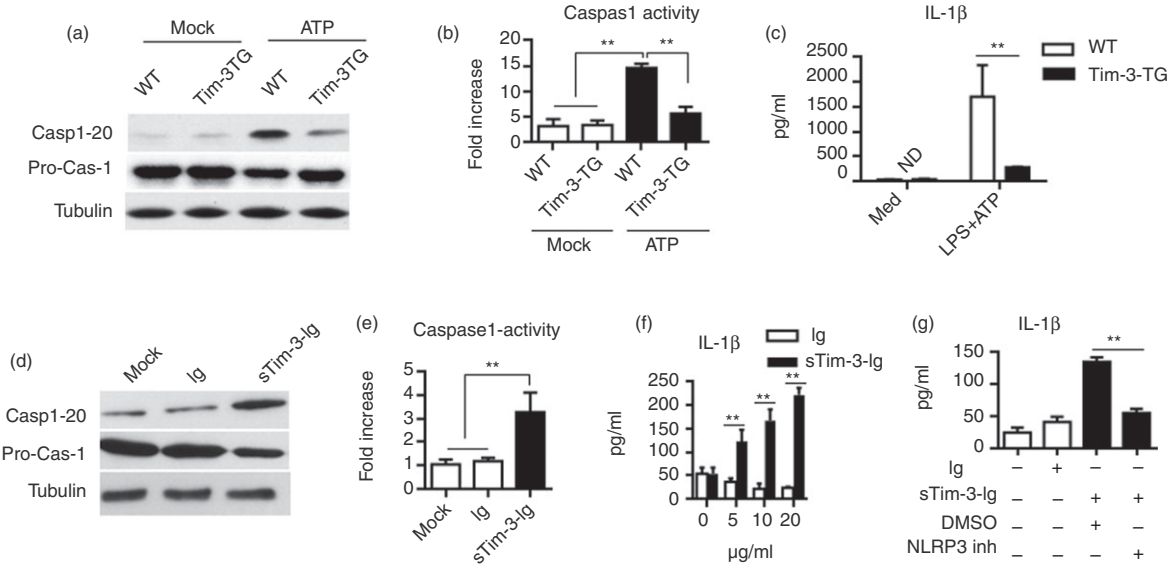

Figure 1. Tim-3 inhibits NLRP3 inflammasome activation. (a–c) Peritoneal macrophages isolated from wild-type (WT) or Tim-3 transgenic (Tim-3-TG) C57BL/6 mice were primed with lipopolysaccharide (LPS) for 6 hr and then either left unstimulated (Mock) or stimulated with 5 mM ATP (30 min). Then (a) macrophages were lysed and examined for Pro-caspase-1 and casp1-p20 levels by Western blotting, (b) caspase-1 activity in the lysate was measured as described in the Materials and methods, and (c) interleukin1β (IL-1β) levels in the culture medium were measured by ELISA. (d–g) J774 cells were incubated with 10 μg/ml of sTim-3-Ig or Ig for 24 hr, or left unstimulated (Mock), then (d) Pro-caspase-1 and casp1-p20 levels and (e) caspase-1 activity in the cell lysate and (f) IL-1β levels in the culture medium were measured as above. (g) J774 cell were incubated with 10 μg/ml of sTim-3-Ig or Ig for 24 hr, or left unstimulated in the presence or absence of NLRP3 inhibitor MCC950 (8 nM), then IL-1β levels in the culture medium were measured as above. In (a) and (c), the experiment was repeated three times with similar results. In (b), (d), (e), (f) and (g) the data shown are representative of three independent experiments, each performed in triplicate.**$P < 0{\cdot}01$.

blocked the LPS-induced increase in NLRP3 mRNA levels (Fig. 2a) and a significantly lower increase in IL-1β mRNA levels (Fig. 2d), whereas, in the macrophage cell lines RAW264.7 (Fig. 2b, e) and J774 (Fig. 2c, f), Tim-3 blockade using different concentrations of sTim-3-Ig (0–20 μg/ml) resulted in a dose-dependent increase in NLRP3 mRNA levels (Fig. 2b, c) and IL-1β mRNA levels (Fig. 2e, f). It is known that the NF-κB pathway is involved in the priming of the NLRP3 inflammasome and promotes expression of NLRP3 and IL-1β. To examine whether Tim-3 inhibited expression of NLRP3 and IL-1β by inhibiting NF-κB activity, J774 cells were incubated for 12 hr with sTim-3-Ig or control Ig with and without the NF-κB inhibitor Bay11-7082 (50 nM), then NLRP3 and IL-1β mRNA levels were measured and the results showed that NF-κB inhibition significantly reduced the Tim-3 blockade-induced increase in NLRP3 (Fig. 2g) and IL-1β (Fig. 2h) mRNA levels.

We then examined whether Tim-3 inhibited NRLP3 expression at the protein level. As shown in the left panels of Fig. 3(a–c), when peritoneal macrophages from WT and Tim-3-TG mice were incubated with LPS for 6 hr and examined by Western blotting, the Tim-3-TG cells exhibited much lower levels of NLRP3, pro-IL-1β and phosphorylated (p) NF-κB p65. In addition, as shown in the right panels of Fig. 3(a–c), when J774 cells were incubated with sTim-3-Ig or control Ig for 24 hr, those incubated with sTim-3-Ig expressed much higher levels of NLRP3, pro-IL-1β and p-NF-κB p65. Finally, to examine whether Tim-3 signalling inhibited NLRP3 and pro-IL-1β protein expression through the NF-κB pathway, J774 cells were incubated with sTim-3-Ig or control Ig with or without Bay11-7082 and, as shown in Fig. 3(d), NF-κB inhibition blocked the Tim-3 blockade-induced up-regulation of NLRP3 (left panel) and pro-IL-1β (right panel). The Western blot results in Fig. 3 were quantified by densitometry and are shown in the Supplementary material (Fig. S2). These data show that Tim-3 inhibits the expression of NLRP3 and IL-1β in macrophages by inhibiting NF-κB activity.

Tim-3 inhibits ATP release by, K^+ efflux from, and ROS production by macrophages

Extracellular ATP stimulates the NLRP3 inflammasome to activate caspase-1, leading to processing of pro-IL-1β.[26] To examine how Tim-3 regulates activation of the NLRP3 inflammasome, we first examined whether it was involved in regulating ATP release by macrophages by incubating J774 cells with 0, 10, or 20 μg/ml of sTim-3-Ig

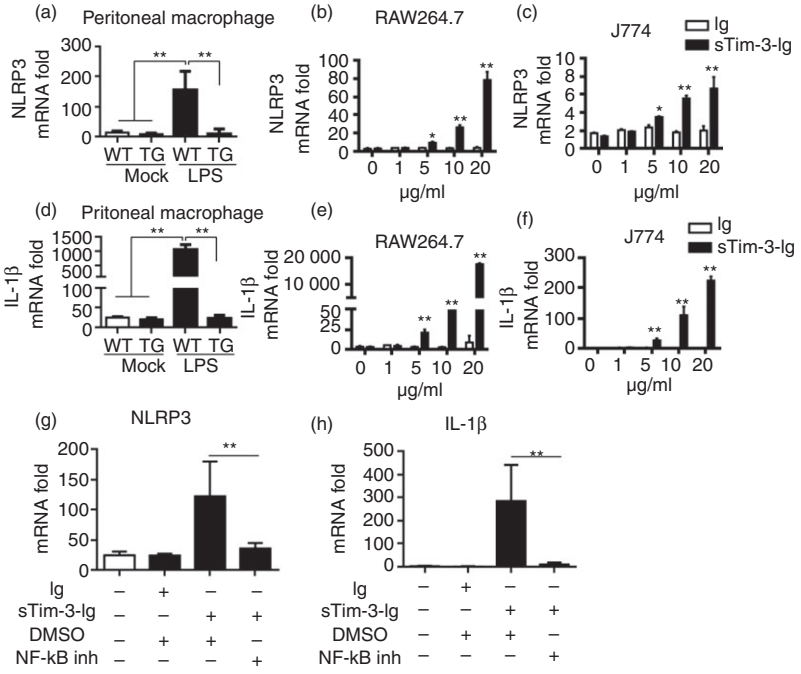

Figure 2. Tim-3 inhibits NLRP3 mRNA expression by inhibiting nuclear factor-κB (NF-κB). (a and d) Peritoneal macrophages from wild-type (WT) and Tim-3-TG mice were incubated with lipopolysaccharide (LPS) (1 μg/ml) for 6 hr or left untreated (mock), then (a) NLRP3 mRNA levels or (d) interleukin-1β (IL-1β) mRNA levels were measured by real-time PCR. (b, c, e and f) RAW264.7 cells (b) and (e) or J774 cells (c) and (f) were incubated with 0–20 μg/ml of sTim-3-Ig or Ig for12 hr, then NLRP3 mRNA levels (b and c) and IL-1β mRNA levels (e) and (f) were measured by real-time PCR. (g) and (h) J774 cells were incubated with 10 μg/ml of sTim-3-Ig or Ig in the presence of the NF-κB inhibitor Bay11-7082 or DMSO control for 12 hr, then NLRP3 mRNA levels (g) and IL-1β mRNA levels (h) were measured by real-time PCR. The data shown are representative of three independent experiments, each performed in triplicate.*$P < 0{\cdot}05$, **$P < 0{\cdot}01$.

or control Ig for 45 min, then measuring ATP levels in the culture medium. As shown in Fig. 4(a), sTim-3-Ig caused a dose-dependent increase in ATP levels in the medium. To test whether the sTim-3-induced ATP is involved in NLRP3 inflammasome activation, a P2X7 ATP receptor inhibitor was added into the cultured J774 cells in the presence of sTim-3. The data in Fig. 4(b) showed that blockade of ATP receptor reversed sTim-3-induced IL-1β production, demonstrating that sTim-3-induced ATP contributes to NLRP3 inflammasome activation.

It is known that ATP opens a large pore that allows the passage of monovalent cations and other small molecules, which results in many cellular events including cytosolic K^+ efflux and ROS production.[27–29] We therefore examined whether Tim-3 was also involved in regulating K^+ efflux from, and ROS production by, macrophages. J774 cells were incubated with 10 μg/ml of sTim-3-Ig or Ig control for 48 hr in the presence of different concentrations of KCl (0–150 mM) to block K^+ efflux, then IL-1β levels in the medium were measured by ELISA. As shown in Fig. 4(c), K^+ dose-dependently inhibited sTim-3-induced IL-1β secretion by the cells. In addition, J774 cells were incubated for 1 hr with sTim-3-Ig or Ig control or for 30 min with 5 mM ATP as a positive control, then intracellular K^+ was measured using the K^+-sensitive fluorophore APG-2 and flow cytometry. The results in Fig. 4(d) show that intracellular K^+ levels were decreased following Tim-3 blockade. These data suggest that Tim-3 signalling may inhibit NLRP3 inflammasome activation by inhibiting ATP release and K^+ efflux.

We also examined the effect of Tim-3 blockade on ROS production by macrophages. Figure 4(e) shows that, when J774 cells were incubated with sTim-3-Ig or Ig control for 3 hr, then stained for ROS levels and examined by flow cytometry, blockade of Tim-3 by sTim-3-Ig increased ROS levels in the cells. In addition, Fig. 4(f) shows that, when peritoneal macrophages from WT and Tim-3-TG mice were stimulated with LPS for 6 hr, then with ATP for another 30 min and cellular ROS levels were examined as above, cells from Tim-3-TG mice contained lower ROS levels than those from WT mice. To test whether Tim-3 inhibited NLRP3 inflammasome activation by decreasing ROS production, J774 cells were incubated for 48 hr with either medium or sTim-3-Ig in the presence or absence of the ROS inhibitor *N*-acetylcysteine (NAC), then IL-1β levels in the medium were measured. As shown in Fig. 4(g), the sTim-3-Ig-stimulated increase

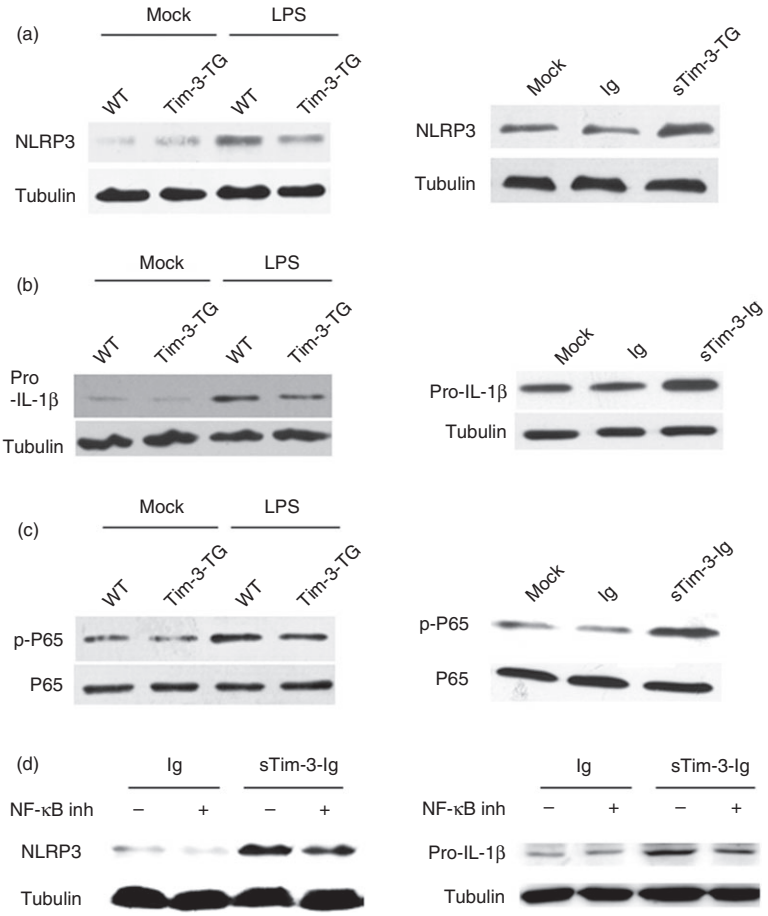

Figure 3. Tim-3 inhibits NLRP3 and pro-interleukin-1β (IL-1β) protein expression by inhibiting nuclear factor-κB (NF-κB). (a–c) Peritoneal macrophages from wild-type (WT) and Tim-3-TG C57BL/6 mice (left panels) were left untreated or incubated with lipopolysaccharide (LPS) (1 μg/ml) for 6 hr or J774 cells were left untreated or incubated with 10 μg/ml of sTim-3-Ig or Ig for 24 hr (for NLRP3) or 30 min (for NF-κB) (right panels), then levels of NLRP3 (a), pro-IL-1β (b) or NF-κB p65 (c) were analysed by Western blotting. (d) J774 cells were incubated with 10 μg/ml of sTim-3-Ig or Ig in the presence or absence of the NF-κB inhibitor Bay11-7082 for 24 hr, then NLRP3 levels (left panel) and pro-IL-1β levels (right panel) were analysed by Western blotting. The experiments were repeated three times with similar results.

in IL-1β secretion was significantly inhibited by NAC. Together, these data suggest that Tim-3 signalling inhibits activation of the NLRP3 inflammasome by inhibiting ATP release, K^+ efflux and ROS production.

Y256 and Y263 in the C-terminal region of Tim-3 are required for the inhibitory effect of Tim-3 on NLRP3 inflammasome activation

Tim-3 did not contain evident inhibitory motif. The molecular mechanisms by which Tim-3 transduces inhibitory signalling remain unclear. It is believed that the intracellular tail of Tim-3 contains a highly conserved tyrosine-containing src homology 2 (SH2) -binding motif in which tyrosine residues Y256 and Y263 within this motif can be constitutively phosphorylated and play critical roles in Tim-3 signalling.[30,31] To determine whether these two residues were required for the inhibitory effect of Tim-3 on NLRP3 inflammasome activation, J774 cells transfected with control Tim-3 or the Y256A Y263A Tim-3 mutant were incubated with LPS for 6 hr, then NLRP3, pro-IL-1β and casp1-p20 levels in the cells and IL-1β levels in the medium were measured. Compared with vector-transfected J774 cells, Tim-3-transfected cells showed reduced LPS-induced expression of NLRP3 and IL-1β at both the mRNA level (Fig. 5a,b) and protein level (Fig. 5c), but the double mutant-transfected cells did not. In addition, when J774 cells were incubated with LPS and ATP, the Tim-3-transfected cells showed less IL-1β secretion (Fig. 5d) and much lower casp1-p20 protein levels (Fig. 5e), whereas the double mutant-transfected cells did not. This mutagenesis study showed that Y256 and Y263 are required for the inhibitory effects of Tim-3 on NLRP3 inflammasome activation. Western blot results in (Fig. 5c, e) were quantified by densitometry and are shown in the Supplementary material (Fig. S3).

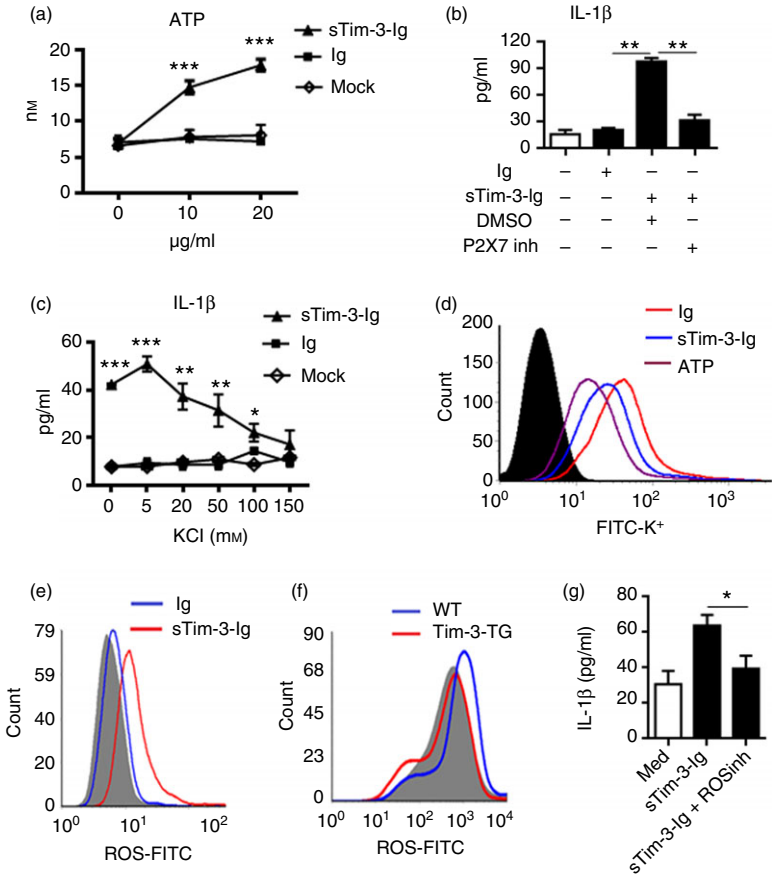

Figure 4. Tim-3 inhibits ATP release, K^+ efflux, and reactive oxygen species (ROS) production. (a) J774 cells were incubated with 0 (mock), 10 or 20 μg/ml of sTim-3-Ig or Ig for 45 min, then ATP levels in the culture medium were measured. (b) J774 cells were incubated with 10 μg/ml of sTim-3-Ig or Ig or left untreated for 48 hr in the presence of P2X7 ATP receptor inhibitor (300 nM) for 24 hr, then interleukin-1β (IL-1β) levels in the culture medium were measured by ELISA. (c) J774 cells were incubated with 10 μg/ml of sTim-3-Ig or Ig or left untreated (mock) for 48 hr in the presence of 0–150 mM KCl then IL-1β levels in the culture medium were measured by ELISA. (d) J774 cells were incubated with 10 μg/ml of sTim-3-Ig or Ig for 1 hr or with 5 mM ATP for 30 min, then intracellular K^+ levels were examined by flow cytometry as described in the Materials and methods. Untreated J774 cells showed similar intracellular K^+ level as that of Ig group (data not shown). (e) J774 cells were incubated with 10 μg/ml of sTim-3-Ig or Ig for 3 hr, then cells were stained for ROS and examined by flow cytometry. Untreated J774 cells showed similar ROS level as that of Ig group (data not shown). (f) Peritoneal macrophages isolated from wild-type (WT) and Tim-3-TG mice were incubated with 1 μg/ml of lipopolysaccharide (LPS) for 6 hr, then 5 mM ATP was added for 30 min, then cells were stained for ROS and examined by flow cytometry. LPS treatment alone of J77 cells did not alter the activity of ROS (data not shown). (g) J774 cells were incubated for 48 hr with 10 μg/ml of sTim-3-Ig in the presence or absence of the ROS inhibitor NAC (20 mM), then IL-1β levels in the culture medium were measured by ELISA. In (a), (b) and (f), the data shown are representative of three independent experiments, each performed in triplicate.*$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$. In (c–e), the experiment was repeated three times with similar results.

Tim-3 protects against peritonitis by inhibiting NLRP3 inflammasome activation

We then examined the effects of Tim-3 signalling on NLRP3 inflammasome activation *in vivo*. First, we measured Tim-3 levels in the serum of healthy controls and patients with peritonitis, an NLRP3 inflammasome-linked disease and, as shown in Fig. 6(a), found that levels were much higher in the patients with peritonitis. An *in vitro* study showed that incubation of the human macrophage cell lines U937 (Fig. 6b, c) and THP-1(Fig. 6d, e) with sTim-3-Ig resulted in increased NLRP3 mRNA levels and IL-1β mRNA levels, suggesting that the increased soluble Tim-3 protein levels in peritonitis patients may enhance the inflammatory response by increasing NLRP3 inflammasome activation. To further examine the effects of Tim-3 signalling on NLRP3 inflammasome activation *in vivo*, an alum-induced peritoneal inflammatory mouse model was used in which mice were injected intraperitoneally with alum and 200 μg of sTim-3-Ig or Ig control, then, 12 hr later, PECs, neutrophils and monocytes were collected from the PLF (6 ml) and counted. Figure 7(a–c) shows that blockade of Tim-3 *in vivo* promoted

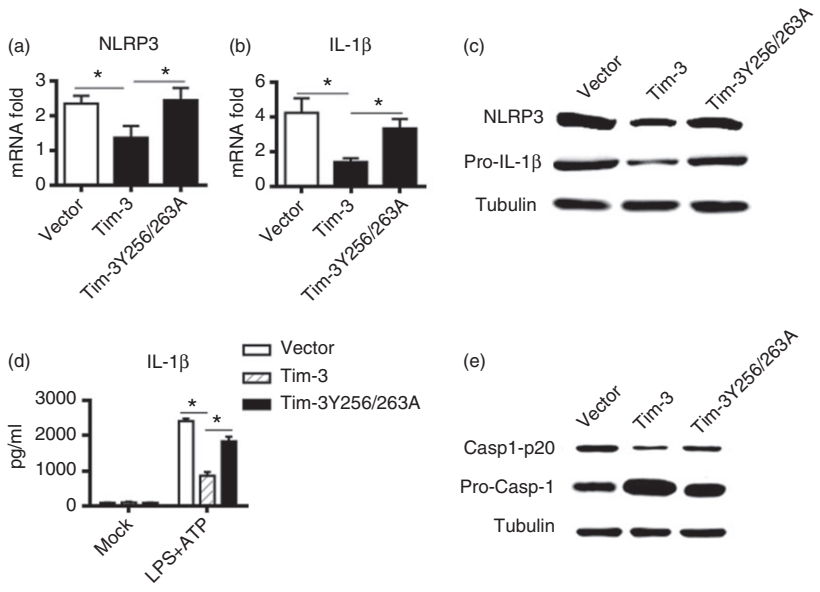

Figure 5. Mutation of residues Y256 and Y263 within the C-terminal tail of Tim-3 to alanine blocks Tim-3-mediated inhibition of NRLP3 inflammasome activation. (a–c) J774 cells were transfected with DNA coding for Tim-3 or the Tim-3 mutation Tim-3-Y256A/Y263A, then, 12 hr later, levels of NLRP3 mRNA (a) or interleukin-1β (IL-1β) mRNA (b) were measured by real-time PCR and levels of NLRP3 or IL-1β protein by Western blotting. (d, e) J774 cells transfected as in (a) and (b) were stimulated with lipopolysaccharide (LPS) (1 μg/ml) for 6 hr, then left untreated (mock) or 5 mM ATP was added for 30 min, then the secretion of IL-1β in the supernatant was measured by ELISA (d), and the levels of Pro-caspase-1 and casp1-p20 in the presence of LPS and ATP were measured by Western blotting (e). The data shown are representative of three independent experiments, each performed in triplicate.*$P < 0{\cdot}05$. In (c) and (d), the experiment was repeated three times with similar results.

progression of peritonitis by increasing the number of PECs, neutrophils and monocytes in the PLF. We also measured IL-1β and IL-6 levels in the PLF and found that blockade of Tim-3 *in vivo* significantly increased IL-1β levels (Fig. 7d) and IL-6 levels (Fig. 7e). Finally, when macrophages were isolated from the PLF by FACS, blockade of Tim-3 signalling *in vivo* was found to result in a marked increase in NLRP3 expression and caspase-1 activity, seen as an increased cas1-p20 cleavage from Pro-caspase-1 in the cells (Fig. 7f), showing that Tim-3 plays a protective role in peritonitis by suppressing NLRP3 inflammasome activation. The Western blot results in Fig. 7(f) were quantified by densitometry and are shown in the Supplementary material (Fig. S4a–c).

To further examine the protective role of Tim-3 in peritonitis, WT and Tim-3-TG mice were injected intraperitoneally with alum, then PECs, neutrophils and monocytes in the PLF were counted 12 hr later. Figure 8 shows that Tim-3 over-expression *in vivo* significantly inhibited the alum-induced increase in the numbers of PECs (a), neutrophils (b) and monocytes (c) and the peritonitis-induced secretion of IL-1β (c) and IL-6 (e). Finally, transgenic over-expression of Tim-3 resulted in a marked decrease in NLRP3 levels and caspase-1 activity (cas1-p20 levels) in macrophages (Fig. 8f), showing that Tim-3 signalling plays a regulatory role in peritonitis by inhibiting activation of the NLRP3 inflammasome. The Western blot results in Fig. 7(f) were quantified by densitometry and are shown in the Supplementary material (Fig. S4d–f).

Discussion

As NLRP3 inflammasome activation is an important triggering event in the development of inflammatory diseases, it must be strictly regulated to protect the host against excessive inflammation. Here, we identified Tim-3 as a new negative regulator of NLRP3 inflammasome activation that might be useful in treating NLRP3 inflammasome-related diseases, such as peritonitis. Tim-3 was found to act in diverse ways, including decreasing the transcription of NLRP3 and the pro-inflammatory cytokine pro-IL-1β by inhibiting NF-κB activity, and reducing NLRP3 inflammasome activation by inhibiting ATP release, K^+ efflux and ROS production. Although the signalling adaptor linking Tim-3 to NLRP3 inflammasome components has yet to be identified, we demonstrated that residues Y256 and Y263 in the C-terminal tail of Tim-3 are required for its inhibitory effects. We also demonstrated an inhibitory role of Tim-3 on NLRP3 inflammasome activation during peritonitis *in vivo*, and, by manipulating theTim-3 pathway, provided a new strategy for treating NLRP3 inflammasome-related diseases.

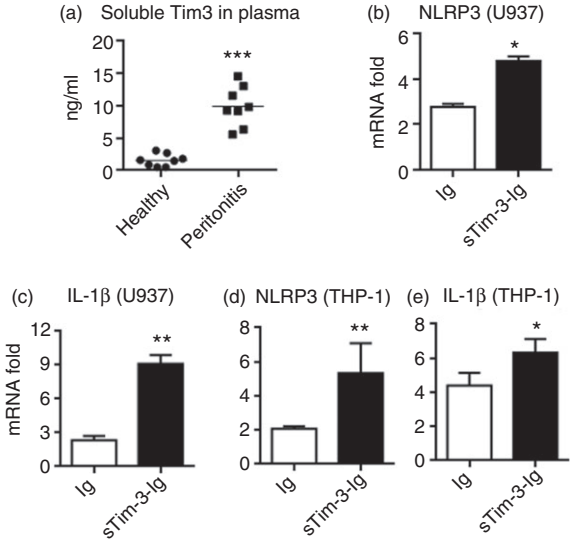

Figure 6. Increased soluble Tim-3 protein level in serum from patients with peritonitis and its possible role in promoting NLRP3 and interleukin-1β (IL-1β) expression in human monocytes/macrophages. (a) Serum samples from patients with peritonitis (n = 8) or healthy controls (n = 8) were examined for sTim-3 levels by ELISA. (b–e) U937 cells (b and c) or THP-1 cells (d and e) were incubated with 10 μg/ml of sTim-3-Ig or Ig control for 12 hr, then NLRP3 mRNA levels (b) and (d) and IL-1β mRNA levels (c) and (e) were measured by real-time PCR. In (b) to (e), the data shown are representative of three independent experiments, each performed in triplicate. *$P < 0{\cdot}05$, **$P < 0{\cdot}01$, ***$P < 0{\cdot}001$.

Tim-3 was initially identified as a negative regulator of T effector cells,[32] but was recently shown to also act as an immune checkpoint inhibitor for innate immunity and to have therapeutic potential.[23,24,33–36] However, it was not known whether NLRP3 inflammasome activation was regulated by Tim-3. To the best of our knowledge, this is the first report showing that Tim-3 negatively regulates NLRP3 inflammasome activation. We also examined the effects of Tim-3 signalling on other inflammasomes, such as NLRC4, AIM2 and NLRP1, and found that it had no effect on inflammasomes other than NLRP3 (data not shown). By showing that Tim-3 specifically controls activation of the NLRP3 inflammasome, we have identified a new mechanism by which Tim-3 contributes to the homeostasis of innate immunity.

Other regulators that suppress NLRP3 inflammasome activation have been identified. For example, type I interferon has been shown to attenuate NLRP3 inflammasome activation in a Stat1-dependent manner,[9] omega-3 fatty acids can negatively regulate NLRP3 inflammasome activation through the G protein-coupled receptors 120 (GPR120) and GPR40,[10] and nitric oxide has been identified as a negative regulator of NLRP3 inflammasome activation.[11] All of these regulators work within the cell and regulate NLRP3 inflammasome activation at either the priming step or the activation step. Here, we identified a membrane protein, Tim-3, that can regulate NLRP3 inflammasome activation at both steps. In addition, as immune checkpoint molecules are now considered potential therapeutic targets for immune disorders, preventing excessive NLRP3 inflammasome activation by manipulating the Tim-3 pathway is a potentially feasible strategy.

Currently, how Tim-3 signalling regulates the activity of innate immune cells is unclear. We previously showed that Tim-3 signalling suppresses LPS-induced macrophage activation by inhibiting NF-κB activity.[20] Here, we demonstrated that Tim-3 also inhibits the expression of NLRP3 and pro-inflammatory IL-1β by inhibiting NF-κB. However, the signalling adaptor linking Tim-3 to NF-κB is still unknown and is under intensive investigation in our laboratory. In addition, we previously found that Tim-3 binds to STAT1.[25] So we also tested whether Tim-3 inhibits NLRP3 and IL-1β expression through STAT1. However, addition of STAT1 inhibitor did not reverse sTim-3-induced NLRP3 and IL-1β expression (data not shown), which suggests that STAT1 is not involved in Tim-3-mediated NLRP3 inhibition. As regards the mechanisms by which Tim-3 suppresses signal 2 of NLRP3 inflammasome activation, we demonstrated that Tim-3 inhibits ATP release, K^+ efflux and ROS production, which are considered triggers of NLRP3 inflammasome activation. We did not examine how ATP release is induced by Tim-3; however, as ATP is involved in energy metabolism and cell activity regulation,[37,38] our results provide a new mechanism by which Tim-3 regulates immune homeostasis. Again, although we demonstrated that Tim-3 signalling suppresses K^+ efflux and ROS production, the underlying mechanisms remain unclear; however, this does not affect the therapeutic potential of Tim-3. Recent findings by Coll and Youm support this idea;[39–41] these authors identified two compounds, MCC950 and BHB, with therapeutic potential, as they suppress K^+ efflux and ROS production by unknown mechanisms and inhibit NLRP3 inflammasome activation.

Next, we examined how Tim-3 signalling inhibits the priming and activation of the NLRP3 inflammasome. No direct interaction between Tim-3 and NLRP3 was seen in a co-immunoprecipitation assay (data not shown). Although the adaptor mediating the inhibitory effect of Tim-3 on NLRP3 inflammasome activation has still to be determined, we showed that residues Y256 and Y263 within the C-terminal tail of Tim-3 are required for transduction of the suppressive signal on NLRP3 inflammasome activation. The molecular mechanism by which Tim-3 signalling suppresses NLRP3 inflammasome activation is now being investigated in our laboratory.

Finally, the clinical significance of our findings was investigated using samples from patients with peritonitis and an animal model of peritonitis. Our results suggested

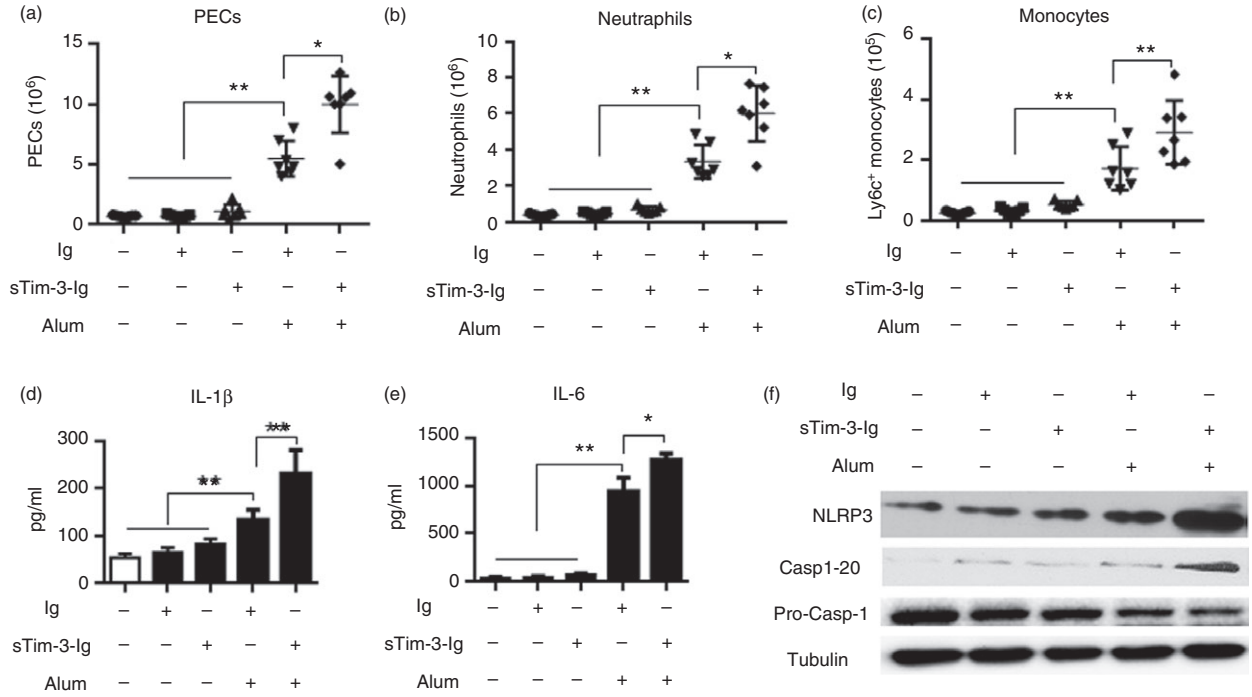

Figure 7. Blockade of the Tim-3 pathway exacerbates peritonitis in a mouse model. Wild-type (WT) mice were injected intraperitoneally with 200 μg of sTim-3-Ig or Ig control and with or without alum, then, 12 hr later, peritoneal lavage fluid (6 ml; PLF) was collected and peritoneal exudate cells (PEC) (a), neutrophils (b) and monocytes (c) stained with specific antibodies were counted and interleukin-1β (IL-1β) levels (d) and IL-6 levels (e) in the PLF were measured by ELISA. (f) Macrophages isolated from the PLF using FACS were examined for NLRP3 levels and Pro-caspase-1 and casp1- p20 levels by Western blotting. In (a–f), cells from naive mice were used as controls. In (a–e), the results, which are representative of two independent assays, are the mean ± SD for six to eight mice per group; $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$. comparing the indicated groups. In (f), the experiment was repeated three times with similar results.

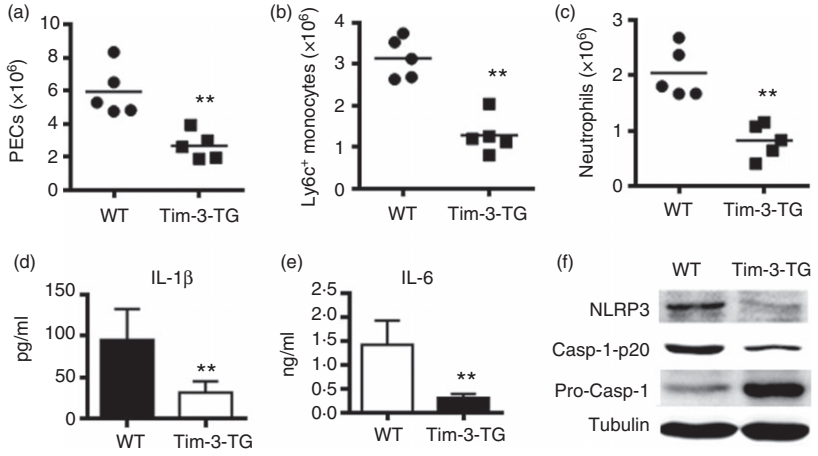

Figure 8. Transgenic over-expression of Tim-3 protects mice from peritonitis. Wild-type (WT) and Tim-3 transgenic mice were injected intraperitoneally with alum, then, 12 hr later, peritoneal lavage fluid (PLF) (6 ml) was collected and peritoneal exudate cells (PECs) (a), neutrophils (b) and monocytes (c) stained with specific antibodies were counted and levels of interleukin-1β (IL-1β) (d) and IL-6 (e) in the PLF were measured by ELISA. (f) Macrophages isolated from the PLF using FACS were examined for NLRP3 levels and Pro-caspase-1 and casp1-p20 levels by Western blotting. In (a–e), the results, which are representative of two independent assays, are the mean ± SD for six to eight mice per group; $^*P < 0{\cdot}05$, $^{**}P < 0{\cdot}01$. In (f), the experiment was repeated three times with similar results.

that increased soluble Tim-3 levels might contribute to the pathogenesis of peritonitis by inducing NLRP3 inflammasome activation, and the *in vivo* data demonstrated that manipulation of the Tim-3 pathway is a potential strategy for treating NLRP3 inflammasome-related diseases, such as peritonitis.

In summary, we have identified a new negative regulator of NLRP3 inflammasome activation. By inhibiting both the priming and activation signals for the NLRP3 inflammasome, Tim-3 contributes to the homeostasis of macrophages. By showing that enhancement of Tim-3 signalling *in vivo* ameliorates peritonitis in an animal model, we have provided a new strategy for treating NLRP3 inflammasome-linked diseases, such as peritonitis, *in vivo*.

Acknowledgements

This study was supported by the key programme of the Beijing Natural Science Foundation (grant 7141007) and National Basic Research Programme 973 grants (2013CB530506 and 2015CB553704), the National Nature and Science Fund (81471540) and the National Key Research and Development Programme of China (2016YFC1202905; AWS16J020).

Author contributions

Wei Wang, QingzhuShi, Shuaijie Dou and Xinhui Shi performed experiments and wrote the manuscript. Xingwei Jang, Ge Li and Zhiding Wang performed the quantitative PCR and Western blot analysis. Renxi Wang and He Xiao performed gene mutation and gene transfection. Chunmei Hou, Yan Li and Jiannan Feng performed flow cytometry analysis and animal experiments. Beifen Shen, Yuanfang Ma, Guojiang Chen and Gencheng Han conceived the idea and edited the manuscript. All authors have read and approved the manuscript.

Disclosures

The authors declare no competing financial interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 (a, b) Peritoneal macrophages isolated from wild-type (WT) or Tim-3 transgenic (Tim-3-TG) C57BL/6 mice were primed with lipopolysaccharide for 6 hr and then either left unstimulated (Mock) or stimulated with 5 mM ATP (30 min).

Figure S2 (a–c) Peritoneal macrophages from wild-type (WT) and Tim-3-TG C57BL/6 mice (left panels) were left untreated or incubated with lipopolysaccharide (1 μg/ml) for 6 hr or J774 cells were left untreated or incubated with 10 μg/ml of sTim-3-Ig or Ig for 24 hr (for NLRP3) or 30 min [for nuclear factor-κB (NF-κB)] (right panels), then levels of NLRP3 (a), pro-interleukin-1β (IL-1β) (b), or NF-κB p65 (c) were analysed by Western blotting. (d) J774 cells were incubated with 10 μg/ml of Tim-3-Ig or Ig in the presence or absence of the NF-κB inhibitor Bay11-7082 for 24 hr, then NLRP3 levels (left panel) and pro-IL-1β levels (right panel) were analysed by Western blotting. The data shown are Western blot results, which were quantified by densitometry using Tubulin as the internal control.

Figure S3 (a, b) J774 cells were transfected with DNA coding for Tim-3 or the Tim-3 mutation Tim-3-Y256A/Y263A, then, 12 hr later, levels of NLRP3 (a) or interleukin-1β (IL-1β) (b) protein by Western blotting. The data shown are Western blot results, which were quantified by densitometry using Tubulin as the internal control. (c, d) J774 cells transfected as in (a) ad (b) were stimulated with lipopolysaccharide (LPS) (1 μg/ml) for 6 hr, then left untreated (mock) or 5 mM ATP was added for 30 min, then the levels of casp1-p20 (c) and Pro-caspase-1 (d) in the presence of LPS and ATP were measured by Western blotting. The data shown are Western blot results, which were quantified by densitometry using Tubulin as the internal control.

Figure S4 (a–c) Wild-type mice were injected intraperitoneally with 200 μg of sTim-3-Ig or Ig control and with or without alum, then, 12 hr later, macrophages isolated from the peritoneal lavage fluid (PLF) using FACS were examined for NLRP3 (a), casp1- p20 (b) and Pro-caspase-1 (c) levels by Western blotting. The data shown are Western blot results that were quantified by densitometry using Tubulin as the internal control. (d–f) Wild-type and Tim-3 transgenic mice were injected intraperitoneally with alum, then, 12 hr later, macrophages isolated from the PLF using FACS were examined for NLRP3(d), casp1-p20 (e) and Pro-caspase-1(f) levels by Western blotting. The data shown are Western blot results, which were quantified by densitometry using Tubulin as the internal control.